STEPAN COMPANY
EFFICACY STUDY OF SINGLE USE IMPREGNATED TOWELETTES
FOR USE AS A SANITIZER FOR FOOD CONTACT SURFACES

Mycoscience Labs GLP Study Protocol # GLP-08-009 rev 00

SPONSOR

Stepan Company
22 Frontage Road
Northfield, IL 60093
PH: 847-501-2242
FAX: 847-501-2455

TEST FACILITY

Mycoscience Labs
25 Village Hill Rd.
Willington, CT 06279
PH: 860-684-0030
FAX: 860-684-0040

PURPOSE OF THE STUDY

To determine the sanitizing activity of a pre-saturated wipe on contaminated 6" x 12" glass and 6" x 12" textured HDPE surfaces at 30 and 60 seconds contact.

TEST SYSTEM AND JUSTIFICATION

Pre-saturated towelettes for sanitizing food contact surfaces will be tested via a modification of the methodology established for sanitizer towlettes in the US Environmental Protection Agency document EPA/AD Method Guidance #02: **Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes** to meet the efficacy data requirements in the US EPA DIS/TSS-4: **Sanitizing rinses (for previously cleaned food-contact surfaces).** The test microorganisms to be used in this study will be ***Escherichia coli* ATCC # 11229** and ***Staphylococcus aureus* ATCC # 6538.**

TEST ARTICLES

1) Canister Stepan Wipe Anywhere, Ref. #: 3401-46 (60 Day Sample)
2) Canister Stepan Wipe Anywhere, Ref. #: 3401-80
3) Canister Stepan Wipe Anywhere, Ref. #: 3401-89

Towel Size: 7" x 8"

TEST SUBSTANCE CHARACTERIZATION

The identity, strength, purity, stability, solubility, and chemical composition of the test material are the responsibility of the sponsor.

CONTROL ARTICLES

Towelettes without active ingredients (e.g. AOAC Neutralizing Broth only)

METHODS

The study will be a modification of the AOAC Germicidal and Detergent Sanitizing Action of Disinfectants, EPA DIS/TSS-4, and the Draft Interim EPA/AD Method Guidance #02: Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes.

PROCEDURE

1.0 The surfaces used in this study will consist of **glass and textured HDPE 6" x 12" surfaces.** The test article will be tested in triplicate against 6" x 12" glass and textured HDPE surfaces inoculated with the test microorganisms.

1.1 The *Escherichia coli* and *Staphylococcus aureus* cultures will be prepared as in AOAC 960.09 D (modified). The surfaces will be inoculated so that they contain a minimum of 7.5×10^7 CFU of the test culture after drying. In order to support claims as a "one-step" cleaner – sanitizer, an organic soil load (5% blood serum) will be incorporated in the inoculum. All inoculated surfaces will be dried at room temperature in a biological safety cabinet for 40 minutes prior to testing.

1.2 One wipe will be used to wipe a single 6" x 12" glass or textured HDPE inoculated surface. The surface will be wiped back and forth in a reciprocal motion so that the entire inoculated surface has been wiped two times. After wiping, the surface section will be allowed to sit for 30 seconds and then will be transferred to an individual sterile bag containing 1,000mL of AOAC neutralizing broth. 30 seconds after wiping the surface section, the wipe will be transferred to a sterile jar containing 200mL of AOAC neutralizing broth. Each bag will be sealed and will be sonicated for 5 minutes along with the jar containing the wipe. Individual surface and wipe extracts will be assayed for surviving numbers of microorganisms using membrane filtration technique. Appropriate aliquots or dilutions thereof will be filtered through individual sterile bacterial retentive filters followed by a 50mL rinse with AOAC neutralizing broth. The membrane filters will be transferred to the surface of Tryptone Glucose Extract Agar (TGEA) plates containing neutralizers, and will be incubated for 48 hours at 35 - 37°C. After the incubation period the plates will be enumerated.
Note: The wipe samples will also be tested at a 60 second contact time per sections 1.0 - 1.2 above.

1.3 A parallel control count will be performed on wipes with the active ingredient(s) omitted. Each inoculated surface section and wipe will be assayed for numbers of microorganisms as in 1.2 above. (Appropriate dilutions of the control extracts will be filtered.)

1.4 All reagents/neutralization media used will be as in AOAC 960.09 A. Note: Neutralization media or neutralizer concentrations may be modified as necessary to provide the adequate level of effectiveness required by the neutralizer validation procedure.

1.5 To demonstrate the absence of residual antimicrobial effect in the neutralizer medium, 100 – 1,000 CFU of the test microorganism will be inoculated to a jar containing a test wipe and 200mL of AOAC neutralizing broth. A control wipe without active in AOAC neutralizing broth will be run for comparison. The jars will be sonicated, and aliquots will be filtered, plated, and incubated as in section 1.2 above. Comparable growth on these plates after incubation will confirm neutralizer effectiveness.

1.6 Surviving organisms will be confirmed as *E. coli* or *S. aureus* by Gram Stain with microscopic and macroscopic examination, and comparison to the positive controls. Additional identification may be performed using the BIOLOG system.

STATISTICAL METHODS: N/A

RESULTS

To be considered valid, results must meet standard effectiveness: at least a 99.999% reduction in the numbers of test microorganisms on the treated surface over that of the parallel control surface in 30 seconds. Results will be reported according to actual count and % reduction over the appropriate parallel control counts.

REPORT

The final report will include an identification of all test articles, summary of the methods used, any modifications to the study, results, summary, and any other pertinent information.

QUALITY ASSURANCE

The Quality Assurance Department will conduct periodic inspections at adequate intervals to ensure the integrity of this study. The Quality Assurance Department will prepare and submit a signed statement to the sponsor with the final report. This GLP study will be conducted according to the Good Laboratory Practice Regulations set forth in 40 CFR: Part 160.

RECORDS

All documentation, data, and final reports derived from this study will be retained in the archives at Mycoscience Laboratories, 25 Village Hill Rd., Willington, CT, 06279.

REFERENCES

1. Draft Interim EPA/AD Method Guidance #02 Dated April 12, 2001: Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes (See Attachment A).

2. Official Methods of Analysis of AOAC International, 17th Edition, 2005, Section 6.3.03. (See Attachment B).

3. EPA DIS/TSS-4: "Efficacy Data Requirements, Sanitizing rinses (for food-contact surfaces)". (See Attachment C).

PROPOSED DATES

Proposed Experimental Start Date: August 14, 2008
Proposed Experimental Completion Date: September 05, 2008

APPROVALS

Study Director: Richard A Fusaro Date: 8-13-08

Mycoscience Labs

Sponsor Approval: Victor Chaney Date: 8/12/08

Stepan Company

Draft Interim Guidance for Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes

April 12, 2001

SUBJECT: Draft Interim Guidance for Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes

The attached draft, interim guidance has been developed for use by AD staff to provide guidance to applicants regarding appropriate methodology that should be utilized when conducting efficacy testing for pre-saturated towelettes. It is a model protocol, which if followed, is likely to provide the type of testing required by the Agency.

This document addresses the Agency's recommendations for evaluating the non-residual sanitizing efficacy of antimicrobial products (specifically pre-saturated towelettes) after application to hard, inanimate surfaces with which food may come in contact.

Specifically, the guidance provides details on the following: 1) purpose and scope of the guidance document, 2) test substance, 3) test methods, 4) reporting of data, 5) test standard, (which includes discussion of the test organisms, procedure, organic soil load, single pack towelettes versus roll of towelettes, towelette size and treatment surface area, and data generation), and 6) performance standard.

This draft, interim guidance should be followed when evaluating efficacy protocols for products of this type. This document may be released to the public when requested. If you have any questions, please contact your branch chief, team leader, or Laura Morris-Bailey.

Attachment

EPA/AD/Method Guidance #02 April 12, 2001

Draft Interim Guidance for AD Staff

Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes

1. Purpose and Scope

The purpose of this document is to provide interim guidance for the evaluation of the sanitizing efficacy of antimicrobial products, specifically pre-saturated towelettes, after application to hard inanimate, nonporous surfaces. This approach may be used to substantiate bacterial sanitizing claims for pre-saturated towelettes applied on hard inanimate surfaces in food contact areas (such as counter tops in restaurants, kitchen cabinets, etc.). This guidance is limited to single use towelettes in both commercial and residential environments. Note: This guidance does not address products for use on utensils, glasses, food containers, dishes, and food processing equipment.

2. Test Substance

Unless otherwise specified, antimicrobial pesticides are to be tested with the formulation to be offered for sale using the product packaged in the same packaging intended to be marketed. Towelettes are a unique combination of antimicrobial chemical products pre-packaged as a unit in fixed proportions for application. Therefore, the complete products, as packaged in the manner to be offered for sale, must be tested according to the directions for use to insure efficacy as a hard surface sanitizer. The product tested must be from three batches as referenced in Section 5.6. Simulated re-use is not required since the product is intended to be removed from the package, used immediately, and discarded after use.

3. Test Methods

Test antimicrobial products in accordance with the proposed directions for use. Depending upon the type of antimicrobial agent, target microorganisms, and the site to be treated, all tests are to address those factors that would normally be expected to be encountered in the use pattern intended for the product, including, but not limited to, the method of application; the nature of the surface (i.e., hard non-porous surface), item surface to be treated; the presence or absence of soil or other interfering conditions; ambient temperature and exposure period of 30 seconds.

Modification of the standard AOAC Germicidal Spray Products Test, official final method, (Official Methods of Analysis of the AOAC International. Chapter 6, Disinfectants, Official Method 961.02 Germicidal Spray Products as Disinfectants, Seventeenth edition. AOAC International, Suite 500, 481 North Frederick Avenue, Gaithersburg, MD 20877-2417) is appropriate for this scenario. Instead of spraying the inoculated surface of the glass slide (as noted in the AOAC Germicidal Spray Products Test method), the towelette product is tested by wiping the surface of the glass slide with the saturated towelette, and then subculturing the slides after a 30 second exposure time. Liquid expressed from the used towelette needs to be subcultured separately. Subcultures of the liquid expressed from the used towelettes are expected to be negative for growth.

4. Reporting of Data

Systematic and complete descriptions of the tests employed and (see item #3 above) the results obtained are essential for proper review and evaluation of product performance by the Agency. All test reports must include identification of the testing laboratory or organization, when and where the tests were conducted and the name of the person(s) responsible for conducting the tests and those who prepared the study report.

5. Test Standard

The following parameters need to be taken into account when developing efficacy data for sanitizing activity of towelettes used on hard inanimate surfaces:

5.1 Test Organisms

Testing is to be based upon an adequately controlled in-use study or simulated in-use study. At a minimum, the microorganisms that must be tested are Staphylococcus aureus (ATCC 6538) and Escherichia coli (ATCC 11229). Testing also must be undertaken for any additional microorganisms that are claimed on the label. The starting inocula of the test microorganisms must be of sufficient concentration to provide between 75 - 125 x 106 cfu/ml on the parallel control surface.

5.2 Procedure

Based on the claims, a variety of surfaces may be treated with the product. Each of the different types of test surfaces claimed may be used in the efficacy testing of the product (i.e., glass, stainless steel, plastic, and ceramic). At a minimum, the applicant must test:1) a stainless steel or glass surface, and 2) a plastic with a rough surface (i.e., plastic cutting boards). Inoculate the test surface with the challenge microorganisms. After inoculation, the test surface is dried for 40 minutes in an incubator at 30 - 37EC. A "zero-time" bacterial numbers recovery test must be performed to demonstrate the efficiency of the recovery process, and must be reported.

The towelette is removed from its container and handled with sterile gloves. The inoculated surfaces are to be tested by wiping the surfaces with the saturated towelette. The area of the towelette used for wiping is rotated so as to expose a maximum amount of its surface in the course of wiping the contaminated test surface. After wiping the contaminated surface with the towelette, all remaining liquid is to be expressed from the used towelette into an empty sterile container and subcultured separately. Run parallel tests on the towelette (as well as expressed liquid from the used towelette) with the active ingredients omitted in an identical manner to serve as the control.

After the 30 second contact time, recover the test microorganisms by washing the treated surfaces with adequate agitation in an appropriate media or dilution fluid containing appropriate neutralizers. Enumerate microorganisms on appropriate nutrient agar, containing the same neutralizers, by the pour or spread plate technique.

The environmental conditions, such as relative humidity and temperature, employed in the test must also be reported. These conditions must be the same as those likely to be encountered under normal conditions of use.

5.3 Organic Soil Load

For products making one-step sanitization claims, the test surface must have an organic soil load applied to the surface prior to the initial treatment and challenge (at a minimum 5% bovine serum). The organic soil level indicated is considered appropriate for simulating lightly or moderately soiled surface conditions. When the surface to be treated has heavy soil deposits, a cleaning step must be required on the label prior to the application of the antimicrobial agent. In the absence of testing with an organic soil load, a one-step claim cannot be made and a pre-cleaning step is required and must be noted on the label.

5.4 Single Pack Towelettes Versus Roll of Towelettes

There may be more moisture retained in a towelette from single pack towelettes than in a towelette from a roll of towelettes. If the towelette roll container does not remain closed, there is a possibility that the towelettes at the end of the roll may not contain as much moisture as those towelettes at the start of the roll. Therefore, to ensure continued efficacy, the label needs to state that the towelette must be visibly wet (saturated) before use, and that the surface treated must be visibly wet after use.

5.5 Towelette Size and Surface Area

At this time, there are no limitations/restrictions regarding the size of the towelette. The Agency's suggested minimum surface area to be treated per towelette is 2' x 2'. However, the size of the surface area treated must be representative of the area that the towelette will treat effectively and reflective of the surface area to be tested in the study. The size of the surface area to be treated, as demonstrated by the data, must also be stated on the label as the recommended maximum surface area to be treated.

5.6 Data Generation

Three samples, representing three different batches, one of which is at least 60 days old, must be evaluated for efficacy against Escherichia coli (ATCC 11229) and Staphylococcus aureus (ATCC 6538). Testing for additional microorganisms claimed on the label is to be conducted on two batches of product. Tests are to be conducted in triplicate.

6. Performance standard

The product must demonstrate at least a 99.999% reduction in the number of test microorganisms (bacteria) within 30 seconds. The result must be reported according to the actual count and percentage reduction over the control.

Guidance approved as Agency standard April 12, 2001 by the Office of Pesticide Programs/Antimicrobials Division.

6.3.03

AOAC Official Method 960.09 Germicidal and Detergent Sanitizing Action of Disinfectants

First Action 1960
Final Action

(Suitable for determining minimum concentration of chemical that can be permitted for use in sanitizing precleaned, nonporous food contact surfaces. Minimum recommended starting concentration is 2–4× this concentration. Test also determines maximum water hardness for claimed concentrations. As control, check accuracy of hard-water tolerance results with pure C_{14} alkyl dimethyl benzyl ammonium chloride at 700 and 900 ppm (μg/mL) hardness, and pure C_{16} alkyl dimethyl benzyl ammonium chloride [Cetalkonium Chloride], at 400 and 550 (μg/mL) ppm hardness, expressed as $CaCO_3$.)

A. Reagents

(a) *Culture media.*—(*1*) *Nutrient agar A.*—Boil 3 g beef extract, 5 g peptone (from Difco No. 0118 or equivalent; special grades must not be used), and 15 g salt-free agar in 1 L H_2O. Do not use premixed, dehydrated media. Tube, and autoclave 20 min at 121°C. Use for daily transfer of test culture. (*2*) *Nutrient agar B.*—Prepare as above but use 30 g agar. Use for growing test cultures in French square bottles. (*3*) *Nutrient agar (AOAC).*—*See* **955.11A(c)** (*see* 6.1.01). Use for preparing stock culture slants.

(b) *Subculture media.*—(*1*) Use tryptone glucose extract agar (Difco No. 0002), adding 25 mL stock neutralizer, (c),/L. (*2*) Tryptone glucose extract agar (Difco).

(c) *Neutralizer stock solution.*—Mix 40 g Lecithin (Alcolec Granules, American Lecithin, PO Box 1908, Danbury, CT 06813, USA [25–50 kg containers only] or Advanced Lecithin Products, PO Box 677, Danbury, CT 06804, USA), 280 mL polysorbate 80, and 1.25 mL phosphate buffer, (e); dilute with H_2O to 1 L and adjust to pH 7.2. Dispense in 100 mL portions and autoclave 20 min at 121°C.

(d) *Neutralizer blanks.*—For use with ≤200 ppm quaternary ammonium compound. Mix 100 mL neutralizer stock solution, (c), 25 mL 0.25M phosphate buffer stock solution, (e), and 1675 mL H_2O. Dispense 9 mL portions into 20 × 150 mm tubes. Autoclave 20 min at 121°C.

(e) *Phosphate buffer stock solution.*—0.25M. Dissolve 34.0 g KH_2PO_4 in 500 mL H_2O, adjust to pH 7.2 with 1M NaOH, and dilute to 1 L.

(f) *Phosphate buffer dilution water.*—Add 1.25 mL 0.25M phosphate buffer stock solution, (e), to 1 L H_2O and dispense in 99 mL portions. Autoclave 20 min at 121°C.

(g) *Test organisms.*—Use *Escherichia coli* ATCC No. 11229 or *Staphylococcus aureus* ATCC 6538. Incubate 24 and 48 h, respectively. Maintain stock cultures on nutrient agar (AOAC), (a)(*3*), at refrigerator temperature.

B. Resistance to Phenol of Test Cultures

Determine resistance to phenol at least every 3 months by **955.11** (*see* 6.1.01). Resistance of *E. coli* should be equivalent to that specified for *S. typhi* in **955.11D** (*see* 6.1.01) and that for *S. aureus* equivalent to that specified for this organism in **955.12** (*see* 6.1.02); also, use procedures under **991.48A(b)** (*see* 6.2.03) for *S. aureus*.

C. Apparatus

(a) *Glassware.*—250 mL wide-mouth Erlenmeyers; 100 mL graduate; Mohr, serological, and/or bacteriological (APHA specification) pipets; 20 × 150 mm test tubes. Sterilize at 180°C in hot air oven ≥2 h.

(b) *Petri dishes.*—Sterile.

(c) *French square bottles.*—175 mL, flint glass.

(d) *Water bath.*—Controlled at 25°C.

D. Preparation of Culture Suspension

From stock culture inoculate tube of nutrient agar *A*, A(a)(*1*), and make ≥3 consecutive daily transfers (≤30), incubating transfers 20–24 h at 35–37°C. Do not use transfers >30 days. If only 1 daily transfer has been missed, no special procedures are required; if 2 daily transfers are missed, repeat with 3 daily transfers.

Prepare 175 mL French square culture bottles containing 20 mL nutrient agar *B*, A(a)(*2*), autoclave 20 min at 121°C, and let solidify with bottle in horizontal position. Inoculate culture bottles by washing growth from slant with 5 mL phosphate buffer dilution H_2O, A(f), into 99 mL phosphate buffer dilution H_2O, and adding 2 mL of this suspension to each culture bottle, tilting back and forth to distribute suspension; then drain excess liquid. Incubate 18–24 h at 35–37°C, agar side down. Remove culture from agar surface of 4 or more bottles, using 3 mL phosphate buffer dilution H_2O and glass beads in each bottle to suspend growth. Filter suspension through Whatman No. 2 paper prewet with 1 mL sterile phosphate buffer, and collect in sterile tube. (To hasten filtration, rub paper gently with sterile policeman.) Standardize suspension to give average of 10×10^9 organisms/mL by dilution with sterile phosphate buffer dilution H_2O, A(f).

Table 960.09A Percent light transmission at various wavelengths corresponding to bacterial concentrations

% Light transmission with filters, nm							Average bacterial count/mL
370	420	490	530	550	580	650	
7.0	4.0	6.0	6.0	6.0	7.0	8.0	13.0×10^9
8.0	5.0	7.0	7.0	7.0	8.0	9.0	11.5
9.0	6.0	8.0	8.0	8.0	9.0	10.0	10.2
10.0	7.0	9.0	9.0	9.0	11.0	11.0	8.6
11.0	8.0	10.0	10.0	10.0	12.0	13.0	7.7
13.0	9.0	12.0	12.0	12.0	13.0	15.0	6.7

Table 960.09B Preparation of $BaSO_4$ suspensions corresponding to bacterial concentrations

Standard No.	2% $BaCl_2$ solution, mL	1% H_2SO_4 (v/v) solution, mL	Average bacterial count/mL
1	4.0	96.0	5.0×10^9
2	5.0	95.0	7.5
3	6.0	94.0	8.5
4	7.0	93.0	10.0
5	8.0	92.0	12.0
6	10.0	90.0	13.5
7	12.0	88.0	15.0

If Lumetron colorimeter is used, dilute suspension in sterile Lumetron tube to give % *T* according to Table **960.09A**.

If McFarland nephelometer and $BaSO_4$ standards are used, select 7 tubes of same id as that containing test culture suspension. Place 10 mL of each suspension of $BaSO_4$, prepared as indicated in Table **960.09B**, in each tube and seal tube. Standardize suspension to correspond to No. 4 standard.

E. Synthetic Hard Water

Prepare *Solution 1* by dissolving 31.74 g $MgCl_2$ (or equivalent of hydrates) and 73.99 g $CaCl_2$ in boiled distilled H_2O and diluting to 1 L. Prepare *Solution 2* by dissolving 56.03 g $NaHCO_3$ in boiled distilled H_2O and diluting to 1 L. *Solution 1* may be heat sterilized; *Solution 2* must be sterilized by filtration. Place required amount *Solution 1* in sterile 1 L flask and add ≥600 mL sterile distilled H_2O; then add 4 mL *Solution 2* and dilute to 1 L with sterile distilled H_2O. Each mL *Solution 1* will give a water equivalent to ca 100 ppm of hardness calculated as $CaCO_3$ by formula:

$$\text{Total hardness as ppm } (\mu\text{g/mL}) \text{ } CaCO_3 = 2.495 \times \text{ppm } (\mu\text{g/mL}) \text{ Ca} + 4.115 \times \text{ppm } (\mu\text{g/mL}) \text{ Mg}$$

pH of all test waters ≤2000 ppm (μg/mL) hardness should be 7.6–8.0. Check prepared synthetic waters chemically for hardness at time of tests, using following method or other methods described in APHA, *Standard Methods for the Examination of Water and Wastewater* 20th Ed., 1998.

F. Hardness Method

(**a**) *EDTA standard solution.*—Dissolve 4.0 g $Na_2H_2EDTA{\cdot}2H_2O$ and 0.10 g $MgCl_2{\cdot}6H_2O$ in 800 mL H_2O and adjust by subsequent dilution so that 1 mL of solution is equivalent to 1 mg $CaCO_3$ when titrated as in (**c**). Check EDTA solution after preparation or, if commecially purchased, against $CaCO_3$ standard at least every 2 months.

(**b**) *Calcium standard solution.*—1 mL = 1 mg $CaCO_3$. Weigh 1.00 g $CaCO_3$, dried overnight or longer at 105°C, into 500 mL Erlenmeyer and add dilute HCl through funnel until $CaCO_3$ is dissolved. Add 200 mL H_2O, boil to expel CO_2, and cool. Add few drops methyl red indicator and adjust color to intermediate orange with dilute NH_4OH or HCl as required. Transfer quantitatively to 1 L volumetric flask and dilute to volume.

(**c**) *Determination.*—Dilute 5–25 mL test sample (depending on hardness) to 50 mL with H_2O in Erlenmeyer or casserole. Add 1 mL buffer solution (67.5 g NH_4Cl and 570 mL NH_4OH diluted to 1 L with H_2O), 1 mL inhibitor (5.0 g $Na_2S{\cdot}9H_2O$ or 3.7 g $Na_2S{\cdot}5H_2O$ dissolved in 100 mL H_2O), and one or 2 drops indicator solution (0.5 g Chrome Black T in 100 mL 60–80% alcohol). Titrate with EDTA standard solution slowly, stirring continuously, until last reddish tinge disappears from solution, adding last few drops at 3–5 s intervals.

$$\text{Hardness as mg } CaCO_3/\text{L} = (\text{mL standard solution} \times 1000)/\text{mL test sample}$$

G. Preparation of Test Samples

Use composition declared or determined as guide to test sample weight required for volume sterile H_2O used to prepare 20 000 ppm (μg/mL) solution. From this stock dilution, transfer 1 mL into 99 mL of the water to be used in test to give concentration of 200 ppm (μg/mL) . In making transfer, fill 1 mL pipet and drain back into stock solution; then refill, to correct for adsorption on glass. After mixing, discard 1 mL to provide 99 mL of the test water in **H**.

H. Operating Technique

Measure 99 mL water to be used in test, containing bactericide at concentration to be tested, into chemically clean, sterile, 250 mL wide-mouth Erlenmeyer and place in constant temperature bath until it reaches 25°C, or ≥20 min. Prepare duplicate flasks for each germicide to be tested. Also prepare similar flask containing 99 mL sterile phosphate buffer dilution H_2O, **A(f)**, as "initial numbers" control.

Add 1 mL culture suspension to each test flask as follows: Whirl flask, stopping just before suspension is added, creating enough residual motion of liquid to prevent pooling of suspension at point of contact with test water. Add suspension midway between center and edge of surface with tip of pipet slightly immersed in test solution. Avoid touching pipet to neck or side of flask during addition. Transfer 1 mL portions of this exposed culture to neutralizer blanks exactly 30 and 60 s after addition of suspension. Mix well immediately after transfer.

For "numbers control" transfer, add 1 mL culture suspension to 99 mL sterile phosphate dilution H_2O in same manner. In case of numbers control, plants need be made only immediately after adding and mixing thoroughly ≤30 s. (It is advantageous to use milk pipets to add culture and withdraw test samples.)

Plate from neutralizer tube to agar, using subculture medium **A(b)**(*1*) for quaternary ammonium compounds and **A(b)**(*2*) with numbers control. Where 0.1 mL portions are planted, use 1 mL pipet graduated in 0.1 mL intervals. For dilutions to give countable plates, use phosphate buffer dilution H_2O, **A(f)**. For numbers control, use following dilution procedure: Transfer 1 mL exposed culture (1 mL culture suspension transferred to 99 mL phosphate buffer dilution H_2O in H_2O bath) to 99 mL phosphate buffer dilution H_2O, **A(f)**, (*dilution 1*). Shake thoroughly and transfer 1 mL *dilution 1* to 99 mL phosphate buffer dilution H_2O, **A(f)**, (*dilution 2*). Shake thoroughly and transfer 1 mL *dilution 2* to 99 mL phosphate buffer dilution H_2O (*dilution 3*). Shake thoroughly and transfer four 1 mL and four 0.1 mL aliquots from *dilution 3* to individual sterile Petri dishes.

For test samples, use following dilution procedure: Transfer 1 mL exposed culture into 9 mL neutralizer, **A(d)**. Shake and transfer four 1 mL and four 0.1 mL aliquots to individual sterile Petri dishes. For numbers control, use subculture medium **A(b)**(*2*); for tests with qua-

ternary ammonium compounds, use medium A(b)(*1*). Cool agar to solidify, and then invert and incubate 48 h at 35°C before counting.

I. Results

To be considered valid, results must meet standard effectiveness: 99.999% reduction in count of number of organisms within 30 s. Report results according to actual count and percent reduction over numbers control. Counts on numbers control for germicide test mixture should fall between 75 and 125 × 10^6/mL for percent reductions to be considered valid.

J. Sterility Controls

(a) *Neutralizer.*—Plate 1 mL from previously unopened tube.

(b) *Water.*—Plate 1 mL from each type of water used.

(c) *Sterile distilled water.*—Plate 1 mL. After counting plates, confirm that surviving organisms are *E. coli* by transfer to brilliant green bile broth fermentation tubes or lactose broth and EMB agar; confirm *S. aureus* by microscopic examination.

References: *Am. J. Public Health* **38,** 1405(1948).
J. Milk Food Technol. **19,** 183(1956).
Fed. Regist. **21,** 7020(1956).
JAOAC **41,** 541(1958); **56,** 308(1973).

6.3.04

AOAC Official Method 961.02 Germicidal Spray Products as Disinfectants

First Action 1961
Final Action 1964

(Suitable for determining effectiveness of sprays and pressurized spray products as spot disinfectants for contaminated surfaces.)

A. Reagents

Use culture media and reagents specified in **991.47A**(a) and (f) (*see* 6.2.02); **991.48A**(a) (*see* 6.2.03); and **991.49A**(a) and (b) (*see* 6.2.05).

Use as test organisms *Trichophyton mentagrophytes* ATCC No. 9533, prepared as in **955.17D** (*see* 6.3.02), to which has been added 0.02 mL octyl-phenoxy-polyethoxy-ethanol (Triton ×100, Union Carbide Corp.)/10 mL suspension to facilitate spreading, *Salmonella choleraesuis* ATCC No. 10708, maintained as in **991.47A**(b) (*see* 6.2.02), *Staphylococcus aureus* ATCC No. 6538, maintained as in **991.48A**(b) (*see* 6.2.03), and *Pseudomonas aeruginosa* ATCC No. 15442, maintained as in **991.49A**(a) (*see* 6.2.05). Incubate all bacterial cultures for 48 h, except *pseudomonas*.

B. Apparatus

Use apparatus specified in **991.47B**(a), (b), (e), (n), and (o) (*see* 6.2.02), and in addition:

(a) *Capillary pipets.*—0.1 mL, graduated to deliver 0.01 mL. Sterilize in air oven 2 h at 180°C

(b) *Microscope slides.*—Noncorrosive, 25 × 25 mm (1 × 1 in.), or 18 × 36 mm glass slide. Sterilize by placing individual slides in Petri dish matted with 2 pieces 9 cm filter paper (Whatman No. 2, or equivalent) in air oven 2 h at 180°C.

(c) *Bacteriological culture tubes.*—Pyrex, 32 × 200 mm (Bellco Glass, Inc., PO Box B, Vineland, NJ 08360, USA).

(d) *Metal forceps.*—Sharp points, straight, 115 mm long.

C. Operating Technique

Thoroughly shake 48 h nutrient broth cultures of *S. choleraesuis* and *S. aureus* and let settle 10 min. For *P. aeruginosa*, follow preparation of culture under **991.49A**(c) (*see* 6.2.05). With sterile capillary pipet or sterile 4.0 mm loop, transfer 0.01 mL culture onto 1 sq in. sterile test slide in Petri dish and immediately spread uniformly over entire area. Cover dish immediately and repeat operation until 12 slides have been prepared for each organism. (Use 2 slides as control.) Dry all slides 30–40 min at 37°C.

Spray 10 slides for specified time and distance. If no time or distance specified, use 10 s at 1 ft. (30 cm). Hold each slide 10 min, drain off excess liquid, and transfer slide to individual 32 × 200 mm tube containing 20 mL appropriate subculture medium, **955.11A**(d) (*see* 6.1.01), with flamed forceps. Shake culture thoroughly. If broth appears cloudy after 30 min, make subculture to fresh individual tubes of subculture broth. Transfer 2 unsprayed slides, as viability controls, to individual subculture tubes in same manner.

Incubate all tubes used for primary and secondary transfers 48 h at 37°C. Read as + (growth) or – (no growth). Killing of test organisms in 10 of 10 trials is presumptive evidence of disinfecting action.

For procedures to be followed in assuring standard cultures, for *S. choleraesuis*, *see* **991.47A**(b) (*see* 6.2.02), for *S. aureus*, **991.48A**(b) (*see* 6.2.03); for *P. aeruginosa*, *see* **991.49A**(c) (*see* 6.2.05). For *T. mentagrophytes*, *see* **955.17A** and **D** (*see* 6.3.02).

If there is reason to believe that lack of growth in subtransfer tubes is due to bacteriostasis, inoculate all incubated subculture tubes with loop needle inoculation of respective test culture and reincubate. Growth of these inocula eliminates bacteriostasis as cause of lack of growth. If there is question as to possibility of contamination as source of growth in subculture tubes, make gram stains and/or subculture for identification, according to respective test culture.

If fungicidal activity as well as germicidal activity is involved, use test suspension of *T. mentagrophytes* spores, **955.17D** (*see* 6.3.02), and prepare 12 slides, using 0.01 mL standard spore suspension, spraying and subculturing exactly as above. Make subcultures in glucose broth, **955.17B** (*see* 6.3.02), incubating 7 days at 25–30°C.

References: *JAOAC* **44,** 422(1961); **50,** 763(1967).
Soap Chem. Spec. **38**(2), **69**(1962); **61,** 400(1978).

6.3.05

AOAC Official Method 966.04 Sporicidal Activity of Disinfectants

First Action 1966
Final Action 1967

(Suitable for determining sporicidal activity of liquid and gaseous chemicals. Applicable to germicides for determining presence or absence of sporicidal activity against specified spore-forming bacteria in various situations and potential efficacy as sterilizing agent.)

A. Reagents

(a) *Culture media.*—(*1*) *Soil extract nutrient broth.*—Extract 1 lb (454 g) garden soil in 1 L H_2O, filter several times through S&S No. 588 paper, and dilute to volume (pH should be ≥5.2). Add 5 g

Sanitizing Rinses (for previously cleaned food-contact surfaces)

DIS/TSS-4 Jan 30, 1979
EFFICACY DATA REQUIREMENTS
Sanitizing rinses (for previously cleaned food-contact surfaces)

Sanitizers applied to food contact surfaces are defined as incidental food additives under the Federal Food, Drug, and Cosmetic Act, as amended (21 U.S.C. 201 et seq.), and require establishment of a food additive tolerance. Recommendation of a potable water rinse after treatment does not preclude this requirement.

1. **Halide chemical products**. Efficacy of sanitizing rinses formulated with iodophors, mixed halides, and chlorine bearing chemicals must be substantiated with data derived from the AOAC Available Chlorine Germicidal Equivalent Concentration Method.

    i. **Test requirements**. Data from one test on each of 3 samples, representing 3 different batches, one of which is at least 60 days old, against S. typhi are required.

    ii. **Performance standard**. Test results must show product concentrations equivalent in activity to 50, 100, and 200 ppm of available chlorine. (The reference standard is sodium hypochlorite.)

2. **Other chemical products**. Efficacy of sanitizing rinses formulated with quaternary ammonium compounds, chlorinated trisodium phosphate, and anionic detergent-acid formulations must be substantiated with data derived from the AOAC Germicidal and Detergent Sanitizers Method.

    i. **Test requirements**. Data from the test on one sample from each of 3 different batches, one of which is at least 60 days old, against both E. coli and S. aureus are required. When claims for the effectiveness of the product in hard water are made, all required data must be developed at the hard water tolerance claimed.

    ii. **Performance standard.** Acceptable results must demonstrate a 99.999% reduction in the number of microorganisms within 30 seconds. The results must be reported according to the actual count and percentage reduction over the control. The minimum concentration of the product which provides the results required above is the minimum effective concentration.